Beiträge zur Tabakforschung International · *Contributions to Tobacco Research* Volume 20 · No. 2 · June 2002
DOI: 10.2478/cttr-2013-0730

Biochemical Changes in β-Cryptogein-Elicited Tobacco: A Possible Basis of Acquired Resistance*

by

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SUMMARY

β-Cryptogein, a proteinaceous elicitor from the phytopathogenic fungus *Phytophthora cryptogea*, is known to induce leaf necrosis in tobacco and non-specific resistance (expressed in the perinecrotic leaf area) against a wide range of tobacco pathogens. To reveal mechanisms underlying the acquired resistance, biochemical changes in leaves of β-cryptogein-elicited tobacco were followed three, five and ten days after elicitation. The activities of peroxidase, β-1,3-glucanase and β-glucosidase, as well as the patterns of acidic pathogenesis-related (PR)-proteins were determined. The protected part (perinecrotic area) and the non-protected part (distant extra-perinecrotic area) of leaves of β-cryptogein-stem treated tobacco (cv. Xanthi n.c.) were analyzed. Leaves of water-stem treated tobacco served as controls. It was shown that in the protected leaf part β-cryptogein caused significant metabolic shifts early after elicitation, persisting during the whole period studied. An important increase of peroxidase and β-1,3-glucanase activity was recorded. PR-protein components appeared that were absent in the controls. There were negligible changes in β-glucosidase activity. In the non-protected leaf part late and non-significant changes occurred. Taking into account the antimicrobial, regulatory and structure-modifying properties of the biochemical components studied, it may be admitted that β-cryptogein elicited the development of a hostile environment, i.e. a potential for plant resistance against subsequent pathogen invasion. [Beitr. Tabakforsch. Int. 20 (2002) 53–59]

ZUSAMMENFASSUNG

β-Cryptogein, ein proteinartiger Elicitor des phytopathogenen Pilzes *Phytophthora cryptogea* ruft bekanntermaßen Nekrose in Tabakblättern sowie eine unspezifische Resistenz (exprimiert im perinektrotischen Bereich der Blätter) gegenüber einer Vielzahl von Tabakpathogenen hervor. Um die Mechanismen, die der erworbenen Resistenz zu Grunde liegen, näher zu untersuchen, wurden die biochemischen Veränderungen bei Blättern der mit β-Cryptogein behandelten Tabakpflanzen drei, fünf und zehn Tage nach der Induktion beobachtet. Die Aktivität der Peroxidase, β-1,3-Glucanase und β-Glucosidase sowie die Muster saurer „pathogenesis-related" (PR)-Proteine wurden bestimmt. Der geschützte Teil (perinekrotischer Bereich) und der nicht-geschützte Teil (distaler extra-perinekrotischer Bereich) der Blätter der mit β-Cryptogein-behandelten Tabakpflanzen (cv. Xanthi n.c.) wurde untersucht. Als Kontrolle dienten Tabakblätter, die mit Wasser behandelt wurden. Dabei zeigte sich, dass β-Cryptogein im geschützten Blattteil sehr schnell nach der Behandlung umfangreiche metabolische Veränderungen verursachte, die während des gesamten Untersuchungszeitraums andauerten. Es war eine bedeutende Zunahme der Peroxidase- und der β-1,3-Glucanase-Aktivität zu verzeichnen. PR-Proteinkomponenten, die in der Kontrollgruppe fehlten, tauchten auf. Die Veränderungen der β-Glucosidase-Aktivität waren minimal. Im nicht-geschützten Teil der Blätter traten zu einem späten Zeitpunkt nicht signifikante Veränderungen auf. Unter Einbeziehung der antimikrobiellen, regulatorischen und strukturmodifizierenden Eigenschaften der untersuchten biochemischen Bestandteile läßt sich die Schlussfolgerung ziehen, dass β-Cryptogein die Bildung einer feindlichen Umgebung auslöst, das heißt, ein Potential zur Resistenzausbildung der Pflanzen gegenüber möglichen Pathogenen aufbaut. [Beitr. Tabakforsch. Int. 20 (2002) 53–59]

RESUME

La β-cryptogéine, un éliciteur de nature protéique isolé à partir du phytopathogène fongique, *Phytophthora crypto*

*Received: 9th July 2001 – accepted: 20th January

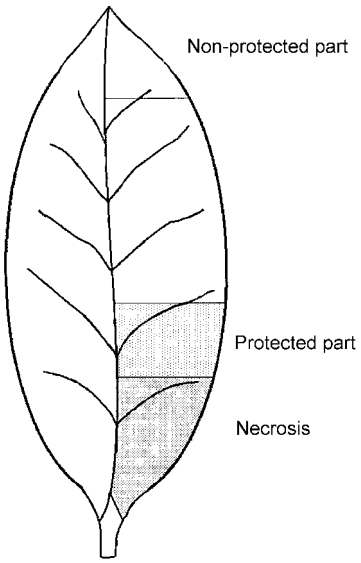

Figure 1. Schematic presentation of a leaf of β-cryptogein-elicited tobacco. Different zones of the leaf are distinguished: necrosis, perinecrotic protected part and distant non-protected part. Corresponding parts of leaves of non-elicited tobacco were used as controls.

gea, est connu pour induire la nécrose des feuilles chez le tabac et une résistance non spécifique (exprimée dans la région foliaire perinécrotique) contre un éventail étendu de pathogènes du tabac. Pour révéler les mécanismes sous-jacents de la résistance acquise, les changements biochimiques dans les feuilles de tabac élicité par la β-cryptogéine ont été suivies trois, cinq et dix jours après l'élicitation. Les activités de la péroxydase, β-1,3-glucanase et β-glucosidase, ainsi que les profils des protéines PR acides ont été déterminés. La partie protégée (région périnécrotique) et la partie non protégée (région périnécrotique distante) des feuilles de tabac traité à la β-cryptogéine (cv. Xanthi n.c.) ont été analysées. Les feuilles de tabac traité à l'eau ont servi comme témoins. Il a été montré que dans la région protégée de la feuille, la β-cryptogéine provoque des modifications métaboliques significatives très tôt après l'élicitation qui persistent pendant toute la période étudiée. Une importante augmentation des activités de la péroxydase et la β-1,3-glucanase est observée. Les composants des protéines PR apparaissent alors qu'elles sont absentes chez les témoins. Des changements négligeables de l'activité de la β-glucosidase sont relevés. Dans la partie non protégée des feuilles, des modifications tardives non significatives se produisent. En prenant en compte les propriétés antimicrobielles, de régulation et de modification de structure des composants biochimiques étudiés, on pourrait admettre que la β-cryptogéine induit la création d'un environnement hostile, c'est à dire un potentiel pour la résistance des plantes contre des pathogènes. [Beitr. Tabakforsch. Int. 20 (2002) 53–59]

INTRODUCTION

A small family of highly conserved 10 kD secretory holoproteins bearing the generic name "elicitins" were isolated from various species of *Phytophthora*: acidic α-elicitins (capsicein and parasiticein), and basic β-elicitins (cryptogein and cinnamomin); these were the first fully characterized proteinaceous fungal elicitors. Application of elicitins to tobacco plant induces expression of both local and systemic resistance to subsequent inoculation with fungal pathogens (10,11,24). β-Cryptogein, an elicitor produced by the fungus *Phytophthora cryptogea*, a non-pathogen of tobacco, when applied on cut tobacco stems, moves basipetally to leaves and induces leaf necrosis and non-specific resistance (expressed in the perinecrotic leaf area) against a wide range of tobacco fungal pathogens: *Botrytis cinerea*; *Sclerotinia sclerotiorum*; *Rhizoctonia solani* and *Erysiphe cichoracearum* (7,11). The protection-inducing effect of β-cryptogein was also demonstrated in tobacco plants expressing a transgene coding for β-cryptogein (32) and in tobacco harboring a fusion between the pathogen inducible tobacco *hsr203J* gene promoter and a *P. cryptogea* gene encoding β-cryptogein (18). In non-induced conditions this transgene is silent, and only becomes expressed (i.e. β-cryptogein is synthesized) upon pathogen infection (18).

The biochemical mechanisms underlying the induction of protection in β-cryptogein-elicited tobacco, suggested as systemic acquired resistance (SAR) (11), are not fully understood. In cell suspension cultures β-cryptogein is shown to induce early events, such as oxidative burst (28,29), K^+ efflux, Ca^{2+} influx, alkalinization and increased conductivity of extracellular medium accompanied by acidification of cytoplasm (8,29), changes in lipid composition (31), lipid peroxidation (28) and protein phosphorylation (34). Binding studies with labelled cryptogein suggest the presence of a single class of binding sites (36). The data point to membranes being a primary target for β-cryptogein interaction with the plant interface followed by triggering of signal transducing pathways.

Little information is available on later events possibly involved in the resistance induced by β-cryptogein. Phytoalexin synthesis and ethylene evolution are reported in β-cryptogein-elicited tobacco (8,28). Other molecules, such as pathogenesis-related (PR)-proteins, oxidative enzymes and glycohydrolases that could contribute to the development of a antimicrobial defense barrier due to their fungitoxic, cell-wall modifying and regulatory properties (14,16,30,33), may also be involved.

Hence, the aim of our work was to investigate the changes in PR-protein patterns, peroxidase, β-1,3-glucanase and β-glucosidase activities in β-cryptogein-elicited tobacco.

MATERIALS AND METHODS

Plants

Tobacco plants, cv. Xanthi n.c. were grown in greenhouse conditions for 45–50 d. Five days before elicitation they were transferred to a chamber (14 h day, 23–24 °C, and 10 h night, 19 °C).

Elicitation

Plants were decapitated above the fifth fully developed leaf. On the cut surface 20 µL of a solution of β-cryptogein

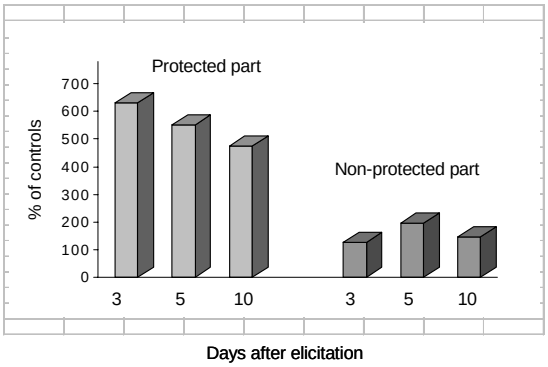

Figure 2. Peroxidase activity in non-protected and protected leaf parts of β-cryptogein-elicited tobacco. Corresponding leaf parts of non-elicited tobacco were used as controls. Data are expressed as percent of the corresponding controls. Values (enzyme units) of the control samples on the third, fifth and tenth day after elicitation are 4.6, 10.2 and 7.3, respectively (protected part), and 7.3, 12.4 and 16.3, respectively (non-protected part). Values are means from three experiments with four replicates each. Standard deviations are less than 10% of the means. Significance of differences from the corresponding controls is given at $P \leq 1\%$.

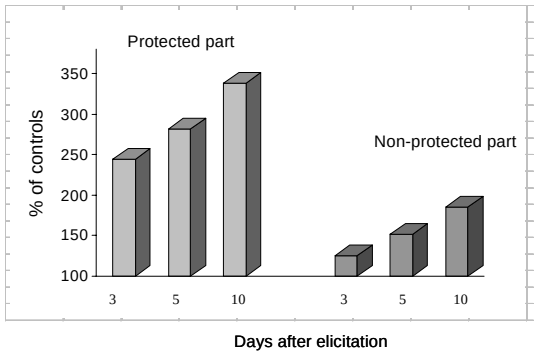

Figure 3. β-1,3-Glucanase activity in non-protected and protected leaf parts of β-cryptogein-elicited tobacco. Corresponding leaf parts of non-elicited tobacco were used as controls. Data are expressed as percent of the corresponding controls. Values (enzyme units) of the control samples on the third, fifth and tenth day after elicitation are 27.0, 28.2 and 29.6, respectively (protected part), and 41.3, 45.5 and 52.5, respectively (non-protected part). For further explanations *see* Figure 2.

(supplied by P. Bonnet) (containing 50 µg in 1 mL water) were applied. Plants treated in the same manner with water were used as controls. Two days after elicitation necrotic zones developed on the leaves below the cutting. These leaves were used for the biochemical analysis.

Sampling

Samples were taken from the perinecrotic zone (2–3 cm around necrosis), named "protected part", and from the distant leaf zone, named "non-protected part" three, five and ten days after elicitation. Control samples were taken at the same intervals from the corresponding zones from leaves of water-treated plants (*see* Figure 1).

Extraction of PR proteins and enzymes

PR-proteins were extracted with a 0.15 *M* phosphate-citrate buffer pH 2.8, containing 0.2% mercaptoethanol (4). Peroxidase was extracted with a 0.05 *M* tris-glycine buffer, pH 8.3, containing 17% succrose in the presence of Dowex 1 × 8 (200–400 mesh), β-1,3-glucanase with a 0.05 *M* sodium acetate buffer, pH 5.0, and β-glucosidase with a 0.15 *M* phosphate-citrate buffer, pH 5.4.

Determination of enzyme activities

Peroxidase (PO; E.C.1.11.1.7) was determined after HERZOG and FAHIMI (17) using 3,3'-diaminobenzidine tetrahydrochloride as a substrate, β-1,3-glucanase (β-1,3-GLU; E.C.3.2.1.39) after PAN *et al.* (27) with laminarine as a substrate, and β-glucosidase (β-GLU; E.C.3.2.1.21) after GIEBEL (15), with *p*-nitrophenyl-β-D-glucopyranoside as a substrate for the enzyme reaction. The enzyme activities were expressed as enzyme units as follows: peroxidase as µmoles H_2O_2 decomposed for 1 min per 1 mg protein; β-1,3-glucanase as µmoles glucose released for 10 min per 1 mg protein; β-glucosidase as µmoles *p*-nitrophenol released for 1 h per 1 mg protein.

Electrophoresis and staining of acidic PR-proteins

A gradient slab system (4–30%) in polyacrylamide gel, pH 8.3, was used and 200 µg protein applied per lane. Coomassie brilliant blue R-250 was used for staining (4).

Statistical analysis

Experiments were repeated three times with separate batches of plants and elicitation, with four replicates per experiment, using leaves of about ten plants per replicate. The Student's test at $P \leq 1\%$ was used for testing the significance of the results. Standard deviations are less than 10% of the means.

RESULTS

Peroxidase activity

Peroxidase activity increased strongly (up to 630% of controls) in the protected leaf part at the earliest stage (three days) after elicitation (Figure 2). The increase was sustained till the end of the elicitation (ten days, 475% of controls). In the non-protected part the increase of peroxidase activity was not so large, varying from 125% to 175% of controls. The values (expressed as enzyme units) of the control samples on the third, fifth and tenth day after elicitation were 4.6, 10.2 and 7.3, respectively (protected part), and 7.3, 12.4 and 16.3, respectively (non protected part). The activity of PO on the third, fifth and tenth day after elicitation in the protected part was 29.0, 55.8 and 34.7 enzyme units, respectively, and in the non-protected part – 9.5, 24.2 and 23.5 enzyme units, respectively.

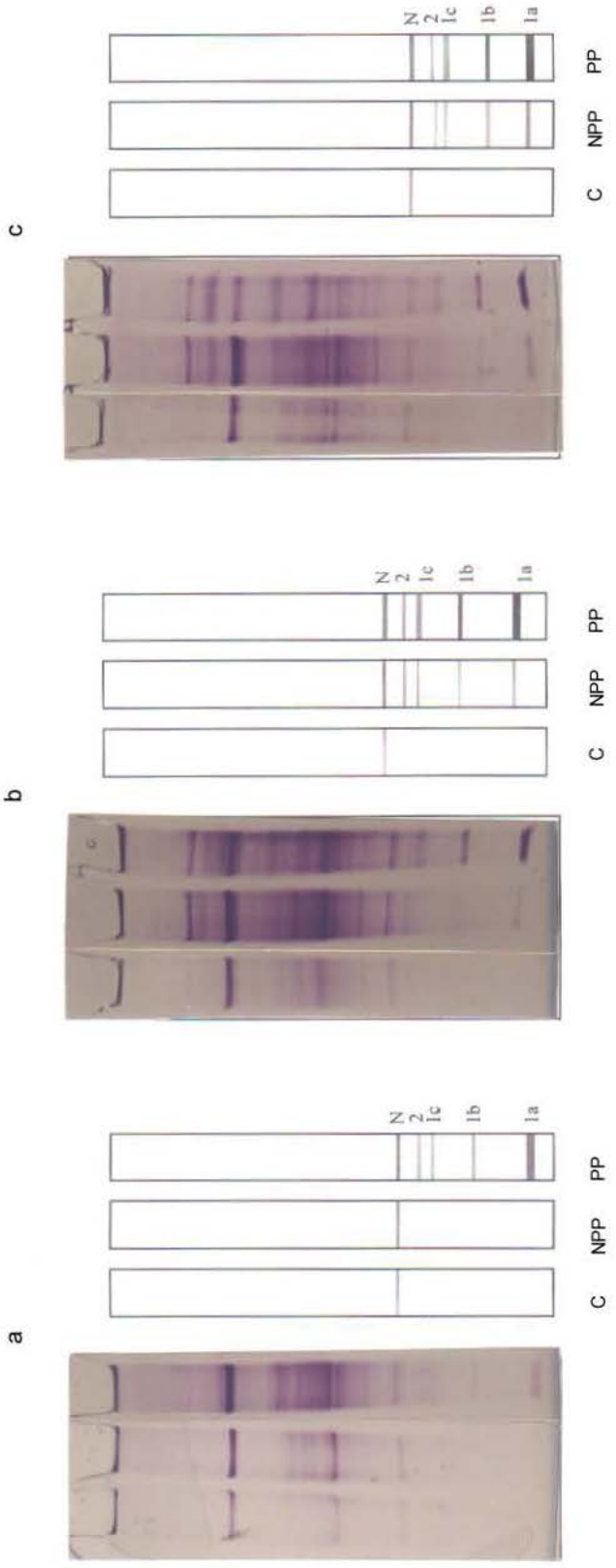

Figure 4. Pattern of acidic PR-proteins in the non-protected part (NPP), protected part (PP) of leaves of β-cryptogein-elicited tobacco and controls (C, leaves of non-elicited tobacco), three (a), five (b) and ten (c) days, respectively, after elicitation. PR-1a, PR-1b, PR-1c, PR-2 and PR-N components are marked as 1a, 1b, 1c, 2 and N.

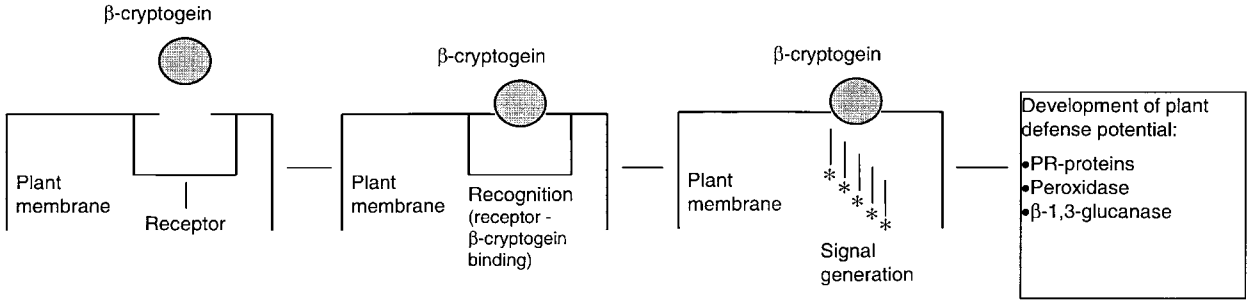

Figure 5. A hypothetical model of β-cryptogein–tobacco interaction

β-1,3-Glucanase activity

A significant rise of activity (244% of controls) was observed in the protected leaf part three days after elicitation (Figure 3). During the period of investigation the enzyme activity was continuously enhanced, reaching 338% of the controls on the tenth day after elicitation. In the non-protected leaf part the changes of the activity were much smaller (125% to 185% of controls).

β-Glucosidase activity

Negligible changes occurred as a result of elicitation (data not shown).

Pattern of acidic PR-proteins

Three days after elicitation four PR-protein components were detected in the protected part (Figure 4). They were identified according to BOL *et al.* (9) as PR-1a, PR-1b, PR-1c (group 1), and PR-2 (group 2a). These PR-proteins were absent in the controls and the non-protected part. During the whole period after elicitation the component PR-N (group 2a) was present in traces in both controls and non-protected part, and in higher amount in the protected zone. Other PR-protein components that have lower mobility were not examined in this study. On the fifth and tenth day after elicitation the intensity of PR-protein bands, especially PR-1a and PR-1b components, increased in the protected zone. In the non-protected part traces of PR-1a, PR-1b, PR-1c and PR-2 appeared on the fifth day; on the tenth day the amount of PR-1a and PR-1b was slightly enhanced.

DISCUSSION

Our experimental results show that β-cryptogein elicitation leads to the development of a complex response in the protected leaf part of tobacco plant involving peroxidase, β-1,3-glucanase and PR-proteins. The different temporal pattern of expression of these components (Figures 2 and 3), suggests their differential role in the protection against microbial invasion. The very rapid and large changes in the protected leaf zone, in contrast to the delayed, smaller changes in the non-protected leaf part, suggests that rate and magnitude of plant responses are important for the effective expression of defense. These data are consistent with evidences from the correlation between expression of SAR and of PR-proteins, β-1,3-glucanase and peroxidase in tobacco (21,33,37) and other plants (30); both pathogens (30,33), pathogen- and plant-derived elicitors (12,23) and synthetic chemical compounds, such as 2-furoic acid, benzothiadiazole (BTH) and β-aminobutyric acid (BABA) (22,30) are reported to coordinately induce SAR and SAR-related molecules.

In our previous work (13) β-glucosidase was shown to be specifically involved in fungal pathogenesis of blue mould (*P. tabacina*) resistance in tobacco. The non-involvement of this enzyme in the events incited by the fungal elicitor β-cryptogein suggests that β-GLU cannot be induced by proteinaceous elicitors, such as β-cryptogein, but probably by specific β-glucans or glucan fragments occurring or released in fungal cell walls.

Peroxidase, β-1,3-glucanase and PR-proteins can play defensive functions (14,16,33), thus serving as a possible biochemical basis of resistance elicited by β-cryptogein. PO is implicated in the control of the active oxygen species pool, including H_2O_2 which is thought to have a central role in plant signalling (26). Moreover, PO/H_2O_2 system is implicated in the regulation of cell wall plasticity by catalyzing lignin biosynthesis and oxidative polymerization of ferulate and tyrosine residues in cell wall components; this may contribute to cell wall cross-linking and fortification, i.e. to building up of a barrier at the plant interface against potential pathogens (14,26). Recent findings (19) lend experimental support to this assumption, showing that β-cryptogein applied to tobacco cell suspension cultures induces loss of digestibility and strengthening of cell walls. β-1,3-GLU is involved in the hydrolysis of fungal cell wall components and the release of active fragments eliciting phytoalexin synthesis in plants (16). Thus, the enzyme may be a constituent of a preformed system contributing to the formation of toxic barrier against subsequent fungal attack.

An impressive body of evidence points to the importance of PR-proteins in plant protection. Although not completely understood, their role may be explained in terms of their lytic action on pathogens, plasma permeabilizing effects and fungitoxicity of some tobacco PR-proteins (1,5,25). The β-1,3-GLU activity of group 2a and 2b acidic tobacco PR-proteins (9) also supports this assumption.

On the basis of available (20,29) and own data, a hypothetical model of β-cryptogein–tobacco interactions, can be proposed (Figure 5). The consequence of events in signal-

transducing cascades downstream from receptor–elicitor binding may be: phosphorylation of proteins → Ca^{2+} influx → H^+-ATPase inhibition → extracellular alkalinization and cytoplasmic acidification → NAD(P)H oxidase activation → active oxygen burst → later events (phytoalexins, PO, β-1,3-GLU, PR-proteins).

CONCLUSION

PO, β-1,3-GLU and PR-proteins in β-cryptogein-elicited tobacco, acting cooperatively, may contribute to the development of a mechanical and chemical defense barrier in tobacco plants against pathogen invasion, i.e. a potentially hostile environment to meet forthcoming pathogen invasion. This hypothesis is substantiated by findings showing that high constitutive levels of PO, β-1,3-GLU and PR-proteins in tobacco (2,3) and other hosts (6,33,35) (occurring normally or following transgene and mutational transformations) are correlated with high levels of resistance to pathogens.

Acknowledgments: The financial support of Tobacco Institute, Bergerac, France, and the Bulgarian National Science Fund (SS 1002/00) is gratefully acknowledged.

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